7-15-2016

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## Recommended Citation

Deochand, D., Mearimanm, J., & Grove, A. (2016). PH-Dependent DNA Distortion and Repression of Gene Expression by Pectobacterium atrosepticum PecS. *ACS Chemical Biology, 11* (7), 2049-2056. https://doi.org/10.1021/acschembio.6b00168

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# pH-Dependent DNA Distortion and Repression of Gene Expression by *Pectobacterium atrosepticum* PecS

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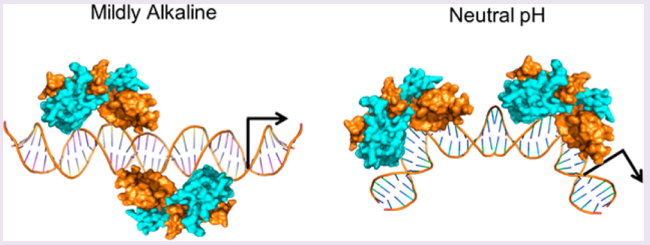
Supporting Information

**ABSTRACT:** Transcriptional activity is exquisitely sensitive to changes in promoter DNA topology. Transcription factors may therefore control gene activity by modulating the relative positioning of −10 and −35 promoter elements. The plant pathogen *Pectobacterium atrosepticum*, which causes soft rot in potatoes, must alter gene expression patterns to ensure growth *in planta*. In the related soft-rot enterobacterium *Dickeya dadantii*, PecS functions as a master regulator of virulence gene expression. Here, we report that *P. atrosepticum* PecS controls gene activity by altering promoter DNA topology in response to pH. While PecS binds the *pecS* promoter with high affinity regardless of pH, it induces significant DNA distortion only at neutral pH, the pH at which the *pecS* promoter is repressed *in vivo*. At pH ∼8, DNA distortions are attenuated, and PecS no longer represses the *pecS* promoter. A specific histidine (H142) located in a crevice between the dimerization- and DNA-binding regions is required for pH-dependent changes in DNA distortion and repression of gene activity, and mutation of this histidine renders the mutant protein incapable of repressing the *pecS* promoter. We propose that protonated PecS induces a DNA conformation at neutral pH in which −10 and −35 promoter elements are suboptimally positioned for RNA polymerase binding; on deprotonation of PecS, binding is no longer associated with significant changes in DNA conformation, allowing gene expression. We suggest that this mode of gene regulation leads to differential expression of the PecS regulon in response to alkalinization of the plant apoplast.




For transcription to occur, bacterial RNA polymerase holoenzyme must bind cognate promoter elements at −10 and −35. Suboptimal spacings between these promoter elements, for example by increasing the spacer from the consensus 17 bp to 19 bp, results in decreased polymerase binding and reduced transcription.[1] Transcriptional regulators may therefore function by modifying promoter DNA conformation such that the relative placement of −10 and −35 promoter elements is altered.

Bacterial transcription factors often respond to environmental signals to effect changes in gene activity. For example, bacterial pathogens must adapt to the hostile environment created by host defenses to achieve successful infection, and the proper temporal expression of virulence genes is key. MarR (Multiple Antibiotic Resistance Regulator) family transcriptional regulators feature prominently in this context as they regulate cellular processes, including virulence, in response to environmental cues.[2−4] Many MarR proteins are encoded divergently from a gene under their control and either repress or activate transcription of the genes in their regulons, a regulatory function that is modulated in the presence of a specific signal or ligand.[4,5]

The enterobacterium *Pectobacterium atrosepticum* (formerly *Erwinia carotovora* subsp. *atroseptica*) is among the most important phytopathogens, responsible for causing soft rot and blackleg disease in potatoes.[6] Soft rot is characterized by maceration of plant cell walls due to secretion of degradative enzymes such as pectate lyases, cellulases, and proteases. Most soft rot *Enterobacteriaceae* belong to the genera *Pectobacterium* and *Dickeya*. The signals to which these bacteria respond in order to effect virulence gene expression range from N-acyl-homoserine lactone (AHL)-dependent quorum sensing to plant-derived factors.[7,8] Taking advantage of natural openings or wounds, the bacteria colonize the plant apoplast, where they encounter the plant's defenses, such as oxidative stress, nutrient limitation, and bactericidal compounds. The mildly acidic (pH 5−6.5) environment of the apoplast is unfavorable for the function of pectate lyases, which have a pH optimum near 8.[9] Soft-rot *Enterobacteriaceae* therefore produce acetoin, which functions to raise the pH of the apoplast.[10] The production of reactive oxygen species (ROS) is another major plant defense strategy; thus neutralization of ROS by antioxidants is crucial for virulence of phytopathogens.[10,11]

Previous studies have shown that *Dickeya dadantii* PecS, a MarR family transcriptional regulator, is one of the primary regulators of virulence gene expression, for example negatively controlling the expression of pectinase genes and production and secretion of the antioxidant indigoidine.[12,13] While *D. dadantii* PecS specifically binds to the intergenic region between divergent *pecS* and *pecM* genes leading to repression of both, the signals that lead to derepression remain to be

Received: February 22, 2016
Accepted: May 23, 2016
Published: May 23, 2016






DOI: 10.1021/acschembio.6b00168
ACS Chem. Biol. 2016, 11, 2049−2056

identified.[13] PecS from the plant pathogen *Agrobacterium tumefaciens* (now designated *A. fabrum*) has also been shown to regulate expression of a divergent *pecM* gene; for *A. fabrum* PecS, urate was shown to be a potent ligand that leads to derepression of gene transcription.[14] Urate is produced as a byproduct during ROS generation by plant xanthine oxidase,[15,16] rationalizing why plant pathogens may detect this compound as a signal for successful host colonization and a contributing trigger of virulence gene expression.

We report here a novel mode of gene regulation by *P. atrosepticum* PecS in which PecS-mediated modulation of promoter DNA topology is controlled by pH. We propose that protonated PecS induces a DNA conformation at neutral pH in which −10 and −35 promoter elements are suboptimally positioned for RNA polymerase binding; on deprotonation of PecS, binding is no longer associated with significant changes in DNA conformation, allowing gene expression. The physiological relevance of this observation is differential expression of the PecS regulon in response to alkalinization of the plant apoplast.

## RESULTS

**Sequence and Structural Analysis.** PecS belongs to the urate-responsive transcriptional regulator (UrtR) subfamily of MarR proteins. UrtR proteins are characterized by an N-terminal extension absent from canonical MarR proteins such as *Escherichia coli* MarR as well as additional signature residues (W24, D68, R75, R101; Supporting Information Figure S1), which may contribute to ligand binding.[14,17,18] To identify bacterial species that conserve this transcription factor, we queried the STRING database[19] with the PecS and PecM protein sequences from *A. fabrum*. STRING predicted conservation of divergent *pecS*−*pecM* gene pairs in a limited set of evolutionarily distant bacterial species (Supporting Information Figure S2). Divergent *pecS*−*pecM* genes were for instance found among the gamma-proteobacterial family *Enterobacteriaceae*, particularly among the genera *Dickeya* and *Pectobacterium*.

The sequence alignment of *P. atrosepticum* PecS with other MarR homologues confirms the UrtR signature (Supporting Information Figure S1). The pairwise alignment shows that *P. atrosepticum* PecS is similar to *D. dadantii* and *A. fabrum* PecS with 48% and 46% sequence identity, respectively. In *D. dadantii*, *A. fabrum*, and *Streptomyces coelicolor*, PecS negatively regulates expression of the *pecM* gene by binding to the *pecS*−*pecM* intergenic region.[14,20,21] PecM is annotated as a permease of the drug/metabolite transporter (DMT) superfamily, which in *D. dadantii* is responsible for efflux of the antioxidant indigoidine;[13,22] pairwise alignment shows 43% identity between *D. dadantii* and *P. atrosepticum* PecM. Examination of the *P. atrosepticum pecS*−*pecM* intergenic region reveals the presence of two pseudopalindromic sequences that resemble the consensus UrtR binding site (TATCTTGANNTNa/gAGATA;[17] Figure 1a).

The *pecS* gene from *P. atrosepticum* was cloned, overexpressed in *Escherichia coli*, and purified to apparent homogeneity; as expected, PecS exists as a dimer in solution as determined by size exclusion chromatography (Supporting Information Figure S3c,d). The secondary structure composition of PecS was predicted by far-UV circular dichroism spectroscopy, yielding a composition that is similar to that of *Deinococcus radiodurans* HucR (Supporting Information Figure S3b).[23] A three-dimensional model of PecS was generated using

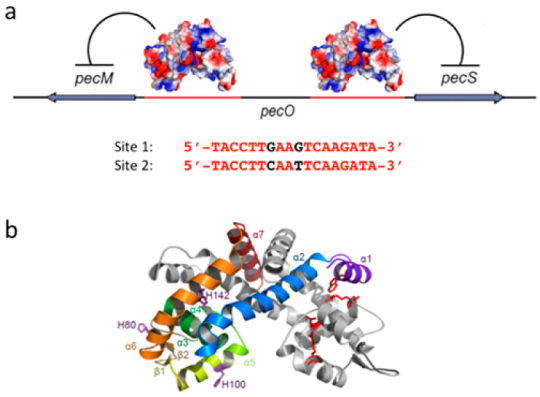



**Figure 1.** *P. atrosepticum* PecS. (a) Divergent *pecM* and *pecS* genes. The intergenic region contains two palindromes separated by 20 bp. One site is 1 bp upstream of the start codon for PecS; the other site is 6 bp upstream of the start codon for PecM. (b). Model of PecS. One subunit is shown in gray and the other in color. The conserved ligand-coordinating residues are shown as red sticks. The histidine residues are shown as magenta sticks.

the HucR structure as a template (Figure 1b). UrtR signature residues shown to coordinate urate in HucR occupy the predicted positions. Notable features of *P. atrosepticupm* PecS include the presence of a histidine in the DNA-recognition helix (H100) as well as a histidine facing the predicted ligand-binding pocket (H142)

**pH-Dependent DNA Binding of PecS.** DNA binding was measured using electrophoretic mobility shift assay (EMSA) using 92 bp (*pecO*) DNA containing the *pecS*−*pecM* intergenic region. PecS bound this DNA with high affinity as evidenced by an apparent dissociation constant ($K_d$) of 1.0 ± 0.1 nM at pH 7.4 (Figure 2a and Supporting Information Figure S4). The Hill

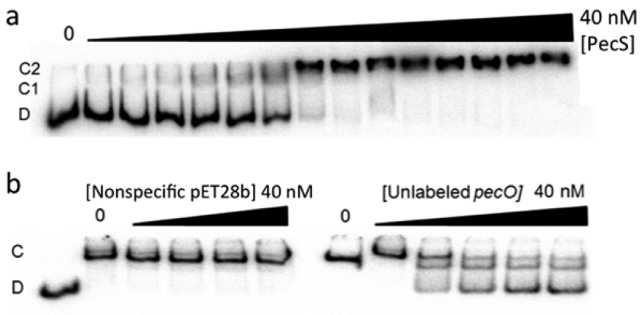



**Figure 2.** DNA binding and specificity of PecS. (a) EMSA showing binding of PecS to the *pecS*−*pecM* intergenic DNA at pH 7.4. [PecS] in lanes 2−15 (nM): 0.03, 0.06, 0.13, 0.25, 0.5, 1.0, 2.0, 3.0, 5.0, 10, 15, 20, 30, and 40. Complexes and free DNA are identified at the left. Experiments were performed in the equilibrium regime ([DNA] < $K_d$). (b) Binding of PecS (2.5 nM) to *pecO* was challenged with increasing concentration (2.5−40 nM) of nonspecific DNA (pET28b) or unlabeled *pecO* DNA.

coefficient ($n_H$) of 1.4 ± 0.1 suggests modest positive cooperativity. PecS formed two complexes with *pecO*, consistent with the predicted presence of two binding sites in the intergenic DNA (Figure 2a). To assess the specificity of DNA binding by PecS, we challenged the PecS−*pecO* complex with excess nonspecific DNA and unlabeled *pecO*. The titration of unlabeled *pecO* with PecS−*pecO* complex showed competition for PecS binding. However, nonspecific DNA had no effect on DNA binding, which demonstrates that PecS binding to


DOI: 10.1021/acschembio.6b00168
ACS Chem. Biol. 2016, 11, 2049−2056

DNA is specific (Figure 2b). When EMSA was performed at pH 8.3, PecS−DNA complexes were unstable and dissociated during electrophoresis (Supporting Information Figure S5).

PecS contains several histidine residues. Considering the p$K_a$ of His, we speculated that His deprotonation might be responsible for the failure to detect a stable PecS−DNA complex at pH 8.3. In addition to three histidine−glutamate repeats at the N-terminus, PecS has four histidines. The PecS model shows H80 at the surface in the loop between helix $\alpha$3 and $\alpha$4, H100 in the DNA recognition helix ($\alpha$5), H142 in helix $\alpha$6, facing the ligand-binding pocket, and H175 near the C-terminus (beyond helix $\alpha$7; Figure 1b and Supporting Information Figure S1). To verify pH-dependent DNA binding by PecS, we performed a filter binding assay as a function of pH (Supporting Information Figure S6). PecS bound to DNA with modestly higher affinity at lower pH, as evidenced by a gradual increase in the apparent dissociation constant ($K_d$) with increasing pH; at pH 5.0, the DNA binding affinity of PecS ($K_d$ = 1.2 ± 0.1 nM) was slightly higher in comparison to pH 8.0 ($K_d$ = 4.3 ± 0.9 nM). These data indicate that DNA binding by PecS is only modestly pH-dependent, but favored at lower pH.

To examine the protonation states of histidines, modification of PecS with diethylpyrocarbonate (DEPC) was carried out at pH 6.6, 7.0, 7.4, and 8.0. Unprotonated histidines react with DEPC and form carbethoxy-histidines. Upon the addition of DEPC, we observed an increase in histidine modification when pH was increased from 6.6 to 7.4 (Figure 3a), suggesting that the extent of histidine modification is pH-dependent and that at least one histidine has a p$K_a$ near 7. The extent of modification of PecS by DEPC at pH 8.0 was less than that at pH 7.4, which might be due to reversibility of the carbethoxylated-histidine at alkaline pH.[24]

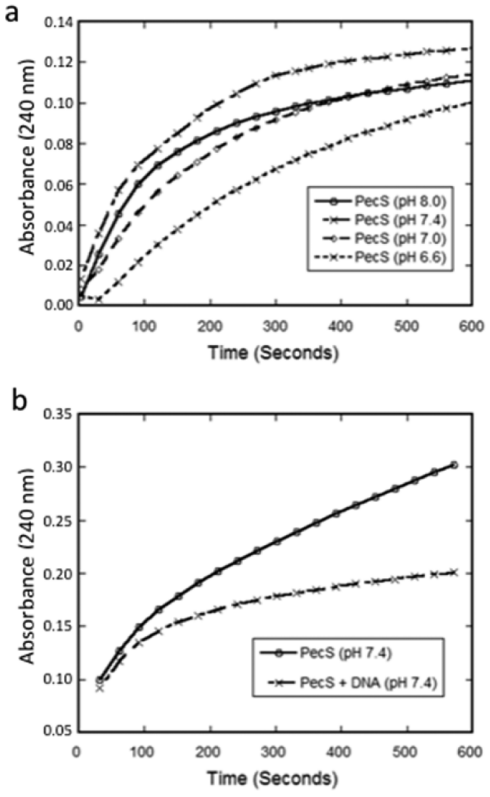



**Figure 3.** Modification of histidines by DEPC. (a) PecS and DEPC were mixed and the absorbance at 240 nm was recorded. (b) DEPC was added to PecS in the presence and absence of DNA containing a single binding site. The higher absorbance observed in comparison to panel (a) is due to presence of Tris, which reacts with DEPC.

PecS was modified with DEPC at pH 7.4 in the presence of 40 bp DNA containing a single PecS binding site. The increase in absorbance at 240 nm was substantially less than for unbound protein (Figure 3b). This indicates that the presence of DNA prevents DEPC from modifying some histidines, either by shielding histidine directly or due to conformational changes that affect DEPC-accessibility of distant residues, and it implicates histidine in DNA binding by PecS.

**PecS Distorts Its Cognate DNA Sites.** To identify cognate sites in the *pecS-pecM* intergenic DNA, we performed DNase I footprinting by the fragment analysis method.[25] A 317 bp fluorescently labeled DNA containing the *pecS−pecM* intergenic region was incubated with or without PecS before digesting it with DNase I. At a PecS concentration of 28 nM, two protected regions, S1 and S2, spanning from −18 to +4 (23 bp) and −57 to −34 (24 bp), respectively, relative to the start codon of PecS were observed at pH 7.4 (Figure 4b,d). At lower concentration of protein (14 nM), protection at site S2 was incomplete, reflecting a modestly preferred binding to site S1 (Figure 4a). Both protected regions correspond to the identified palindromes, with protection starting at the upstream end of each palindrome and extending 6 bp downstream. Notably, hypersensitive cleavage sites flanking each protected region (centered 7 bp from either side of each palindrome) were observed at positions −64, −33, −32, −26, −25, and +6. This is significant, because it suggests that PecS distorts the DNA upon binding.

A similar pattern of protection was observed at pH 8.3 (Figure 4c and Supporting Information Figure S7a). However, while hypersensitive site positions are identical at pH 7.4 and 8.3, hypersensitive cleavage is much less pronounced at pH 8.3 even though protection of both palindromes is complete, suggesting full occupancy (Figure 5). This difference in hypersensitive cleavage suggests a DNA deformation upon PecS binding at pH 7.4 that is virtually absent when PecS binds DNA at pH 8.3.

To further investigate the effect of pH and the role of histidine on DNA binding by PecS, we created two mutants, PecS-H100F and PecS-H142F; H100 is in the DNA recognition helix and might be involved in direct contacts to DNA, and H142 faces the predicted ligand-binding pocket (Figure 1b; for further justification, see Supporting Information).

Consistent with H100 being predicted to be surface-exposed, thermal stability of PecS-H100F was not appreciably different from that of wild-type PecS (measured at pH 7.4; data not shown). Footprinting performed with PecS-H100F (28 nM) at pH 7.4 did not show any protection, only a hypersensitive cleavage at position −69 (Supporting Information Figure S7b). Using higher protein concentration (56 nM), PecS-H100F protection resembled that of wild-type PecS, including the flanking hypersensitive sites (Figure 6a). However, we observed three additional hypersensitive sites at −69, +59, and +106. EMSA with PecS-H100F indicated an approximately 1.5-fold decrease in affinity ($K_d$ = 2.5 nM; Supporting Information Figure S8), suggesting that DNase I footprinting was performed under stoichiometric conditions as for wild-type PecS. Noting that expression of PecS-H100F in *E. coli* was much lower than that of wild-type PecS as judged by protein content in whole


DOI: 10.1021/acschembio.6b00168
ACS Chem. Biol. 2016, 11, 2049−2056

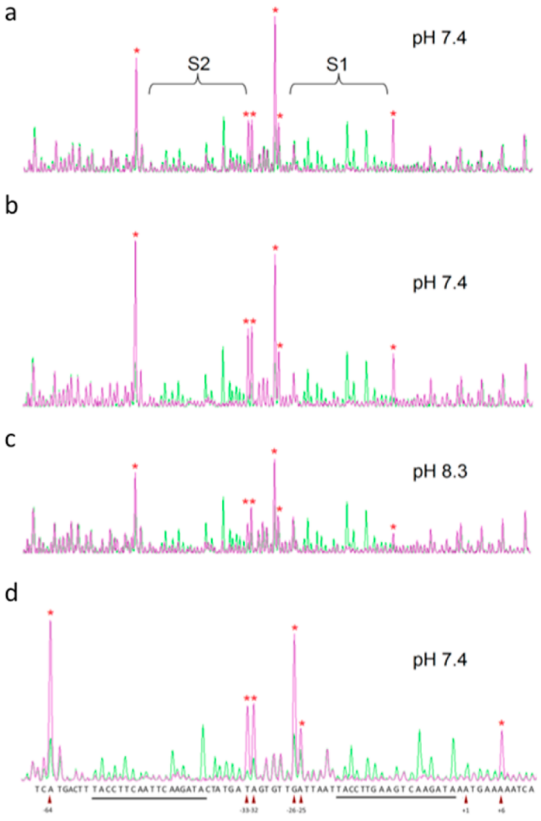



**Figure 4.** DNase I footprint of PecS binding to *pecS*–*pecM* intergenic DNA. (a) DNase I digestion of DNA performed at pH 7.4 without (green) or with PecS (14 nM; magenta). Two protected regions, S1 and S2, are identified. (b) DNase I digestion at pH 7.4 without (green) or with PecS (28 nM; magenta). (c). DNase I digestion at pH 8.3 without (green) or with PecS (28 nM; magenta). The *y* axis (in arbitrary units) is constant in panels a–c, as reflected in equivalent height of peaks at the far left and far right, where cleavage was unaffected by protein addition. (d) Expanded view of protected regions (S1 and S2) from panel b. The DNA sequence is shown, and the palindromes are underlined. The sequence numbering is relative to the start codon of PecS, defined as +1. (a–d) Hypersensitive sites induced by PecS binding to DNA are represented with red asterisks. Experiments were performed under stoichiometric conditions ([DNA] $\gg K_d$).

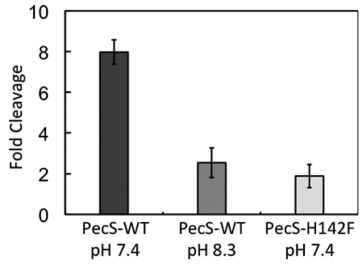



**Figure 5.** Quantitation of hypersensitive cleavage. The relative intensity of cleavage at sites identified by asterisks in Figure 4. Peak heights for all six marked hypersensitive sites were totaled and normalized to the sum of peak height for six peaks not altered by protein binding. Error bars represent standard deviation.

cell lysate (Supporting Information Figure S9), an outcome that would be expected if PecS-H100F bound DNA less specifically and interfered with normal cellular function, we therefore surmise that the additional hypersensitive sites reflect binding to alternate sites.

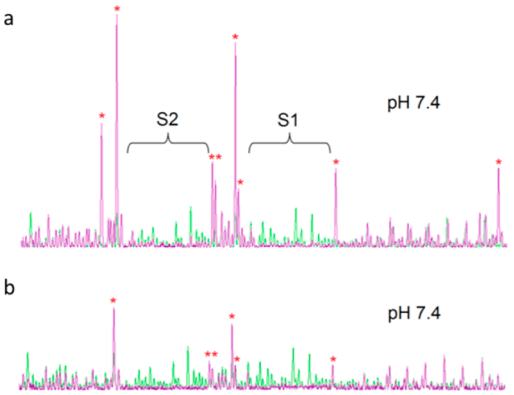



**Figure 6.** DNase I footprint of PecS variants. (a) DNase I digestion without (green) or with PecS-H100F (56 nM; magenta). The two protected regions are identified by brackets; note that the *x*-axis is expanded relative to that shown in Figure 4 to include additional hypersensitive sites. (b) DNase I digestion without (green) or with PecS-H142F (28 nM; magenta). (a,b) Red asterisks indicate the hypersensitive sites. Experiments were performed under stoichiometric conditions ([DNA] $\gg K_d$).

The footprint of PecS-H142F revealed an identical protection pattern to wild type PecS, including preferred protection of site S1 (Supporting Information Figure S7c). Complete protection was observed at the same concentration (28 nM; Figure 6b) required for complete protection by wild-type PecS (Figure 4b). However, hypersensitive cleavage was much less pronounced compared to wild type PecS (Figures 5 and 6b). Taken together, our data indicate that PecS induces significant DNA distortion at neutral pH, but not at mildly alkaline pH. Notably, the H142F substitution yields a mutant protein that does not induce the marked DNA distortions characteristic of wild-type PecS. For wild-type PecS, this suggests that protonation of His142 induces conformational changes that propagate to the DNA-binding motifs and that DNA-binding by protonated PecS requires DNA distortion. By contrast, the disposition of DNA recognition helices in deprotonated PecS (pH ∼ 8) or PecS-H142F is compatible with binding in consecutive DNA major grooves without the need for major DNA distortion. That the H142F substitution imposes changes in protein structure or dynamics is supported by the observation that PecS-H142F exhibits a modestly lower thermal stability at pH 7.4 (45.5 ± 0.6 °C compared to 51.2 ± 1.2 °C for wild-type PecS), consistent with H142 occupying a more buried position near the ligand-binding pocket.

**pH-Dependent Control of Gene Expression.** A transcriptional reporter construct was created in which the gene encoding enhanced green fluorescent protein (EGFP) is under control of the *pecS* promoter (*pecO*). In *E. coli* carrying pACYC184_EGFP_pecO and pET100-PecS in which PecS expression was not induced by IPTG, the cells expressed EGFP at pH 7 (Figure 7a, right panel). However, when PecS expression was induced, no fluorescence was observed, indicating that expression was repressed by PecS (Figure 7b, left panel). Since PecS was expected to respond to urate with attenuated DNA binding as observed for other PecS proteins,[14,20] urate was added to cultures in which PecS expression had been induced. The addition of urate to PecS expressing cells was found to markedly induce the expression of EGFP (Figure 7b, right panel). This suggests that urate acts as a


DOI: 10.1021/acschembio.6b00168
ACS Chem. Biol. 2016, 11, 2049–2056

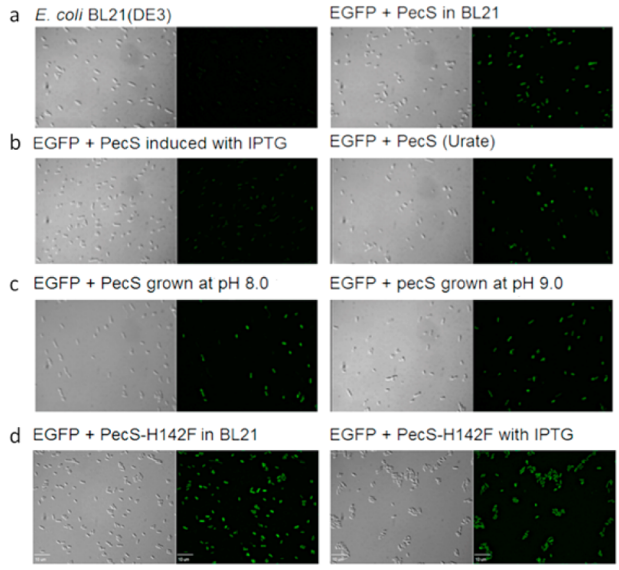



**Figure 7.** Alkaline pH relieves PecS-mediated repression of the *pecS* promoter *in vivo*. Phase contrast (left) and fluorescent micrographs (right) are shown. (a) *E. coli* harboring no plasmids (left column) and *E. coli* transformed with plasmids carrying genes encoding EGFP and PecS (no IPTG induction, right column). (b) *E. coli* transformed with plasmids carrying genes encoding EGFP and PecS induced with IPTG without (left) and with urate (right) at pH 7.4. (c) *E. coli* transformed with plasmids carrying genes encoding EGFP and PecS induced with IPTG at pH 8.0 (left) or at pH 9.0 (right). (d) *E. coli* transformed with plasmids carrying genes encoding EGFP and PecS-H142F without (left) or with IPTG induction of PecS-142F expression at pH 7.4. The scale bar represents 10 $\mu$m and is same for all micrographs.

ligand for PecS *in vivo* and triggers derepression of the *pecS* promoter.

To determine if PecS control of the *pecS* promoter is pH dependent, the strain harboring pACYC184_EGFP_pecO and pET100-PecS was grown at pH 8.0 and 9.0 and PecS expression was induced with IPTG (Supporting Information Figure S9). *E. coli* usually maintains its cytoplasmic pH within a specific range of 7.4−7.8, with ΔpH between extracellular and intracellular pH of ∼0.2 and 0.5 for cells grown at pH 8 and 9, respectively.[26] The presence of EGFP fluorescence suggests that PecS failed to repress the *pecS* promoter when intracellular pH reached ∼7.8 (extracellular pH ∼ 8; Figure 7c), as reflected in fluorescence in both uninduced (Supporting Information Figure S10) and IPTG-induced cultures. These data substantiate DNase I experiments, which show that DNA distortion by PecS is pH-dependent, and they suggest that the difference in extent of DNA distortion correlates with activity of the *pecS* promoter *in vivo*.

To examine whether the H142F mutation impacts *pecS* promoter binding *in vivo*, the strain carrying pACYC184_EGFP_pecO and pET100-PecS-H142F was cultured with and without IPTG at pH 7.4. We observed EGFP fluorescence in both uninduced and induced cultures (Figure 7d). This indicates that His142 is required for repression of the *pecS* promoter by PecS, and it is consistent with the observation that DNA binding by PecS-H142F does not involve significant DNA distortion. An *in vivo* experiment with pET100-PecS-H100F was not performed, due to the low expression of PecS-H100F in comparison to wild type PecS.

**Urate Attenuates DNA Binding by PecS.** DNA binding of UrtR family transcriptional regulators is usually attenuated in the presence of urate.[14,20,27,28] Consistent with this prediction, urate functioned as a PecS ligand *in vivo* (Figure 7b). Further, DNA binding of PecS was significantly attenuated in the presence of urate with an $IC_{50}$ of 7.6 ± 0.1 mM (Supporting Information Figure S11). Based on the $IC_{50}$, an apparent inhibition constant $K_i$ of 2.1 mM was estimated (the 0.5 M Tris included in the reaction buffer to prevent changes in pH on addition of urate dissolved in 0.4 M NaOH reduces the affinity of urate). Evidently, *P. atrosepticum* PecS likewise responds to urate by attenuated DNA binding.

To confirm direct ligand binding, we measured intrinsic tryptophan fluorescence of PecS as a function of urate concentration; the addition of increasing concentration of urate (5−100 $\mu$M) resulted in gradual quenching of intrinsic fluorescence (Supporting Information Figure S12). We also performed a thermal shift assay to monitor the effect of urate on protein stability. The fluorescence intensity of SYPRO Orange fluorescent reporter was measured as a function of temperature. A one step melting transition was observed for unbound protein with a melting temperature of 50.0 ± 0.4 °C (Supporting Information Figure S13 and Table S1). The addition of 10 $\mu$M urate to PecS resulted in a decrease of the melting temperature of ∼4 °C, whereas the addition of higher urate concentration (100 $\mu$M) led to a further decrease in melting temperature ($T_m$ ∼ 45 °C). This suggests that conformational changes in PecS are induced at a micromolar concentration of urate. By comparison, the affinity of urate for *A. fabrum* PecS and *D. radiodurans* HucR is ∼10 $\mu$M.[14,29]

## DISCUSSION

**Physiological Relevance of pH Dependence.** Phytopathogenic bacteria depend on sensing and responding to environmental cues for efficient colonization of host plants, often subverting host defenses by utilizing plant-derived signals to trigger upregulation of virulence genes. MarR family transcriptional regulators mediate such adaptive responses to different environmental stimuli.[2,18] *P. atrosepticum* PecS binds to the intergenic region of *pecS*−*pecM* with high affinity and specificity (Figure 2), and it efficiently represses the *pecS* promoter *in vivo* at neutral pH (Figure 7). This would be consistent with the ability of PecS to prevent premature expression of virulence genes during early stages of colonization. PecS binds to two sites in the *pecS*−*pecM* intergenic region (Figure 4); given that only 62 bp separate the start codons of *pecM* and *pecS*, we infer that PecS binding to either site would repress transcription of both genes.

By comparison, DNase I footprinting of *D. dadantii* PecS on the corresponding *pecS*−*pecM* intergenic DNA revealed two protected regions of 32 and 45 bp, respectively, with each region characterized by a relatively high A+T-content, but no identifiable palindromes.[13,30] For *A. fabrum* PecS, two protected regions were likewise seen, both corresponding to palindromes that resemble the UrtR consensus.[14]

Production of the main pectate lyases in *D. dadantii* is subject to complex regulation. On these genes, the affinity of PecS is ∼10-fold lower than for the site in the *pecS*/*pecM* promoter, and it was not possible to footprint PecS alone.[30,31] Similarly, it is conceivable that *P. atrosepticum* PecS recognizes more degenerate sequences in other gene promoters. For example, the promoters driving expression of the three major pectate lyases PelA, PelB, and PelC encoded by *ECA_RS20105*-*ECA_RS20115* all feature a highly conserved site with consensus sequence AAATTCTAT-TCCAAGGAG, in which


DOI: 10.1021/acschembio.6b00168
ACS Chem. Biol. 2016, 11, 2049−2056

each half-site conserves 3 and 5 bp, respectively, of the consensus PecS site in the *pecS*−*pecM* intergenic DNA.

PecS represses gene expression at neutral pH. This observation supports previous reports that expression of virulence genes such as genes encoding pectate lyases is low at acidic pH. During the initial phase of colonization, there is no production of plant cell-wall-degrading enzymes.[32] Once bacteria colonize the plant intercellular spaces, the onset of pathogenesis begins with alkalization of the plant apoplast,[10] which is necessary as pectate lyase functions above pH 8.0. Thus, we propose that the altered mode of DNA binding by PecS at alkaline pH contributes to a gradual onset of virulence gene expression and therefore pathogenesis of *P. atrosepticum.*

**Modulation of Promoter DNA Topology.** PecS binds DNA with comparable affinity regardless of pH, yet repression of the *pecS* promoter is effectively abrogated at extracellular pH ∼ 8 (where *E. coli* is expected to maintain its intracellular pH ∼ 7.8;[26] Figure 7). Notably, the binding of PecS to DNA introduces marked distortion at cognate sites at neutral pH, as evidenced by the presence of significant hypersensitive sites (Figures 4 and 5). However, an increase in pH is associated with severe attenuation of hypersensitive cleavage, indicating that the DNA distortion induced by PecS in its repressive mode (at pH ∼ 7) is attenuated at pH ∼ 8, where DNA binding does not repress gene activity. The 38 bp distance between the centers of identified palindromes would correspond to ∼3.5 helical turns, which would place two PecS dimers on opposite faces of the duplex, assuming *B*-form DNA. This binding mode might apply at pH 8.3 when little DNA distortion is induced on DNA binding (Figure 8). The significant distortion induced at neutral pH may involve DNA bending or unwinding and may force a different position of PecS dimers relative to each other. We speculate that PecS may impose conformational changes in promoter DNA that adversely affect the ability of RNA polymerase to bind or initiate transcription, but only at neutral pH (Figure 8). At the permissive pH near 8, PecS binding does not lead to large conformational changes, and productive recruitment of RNA polymerase may occur.

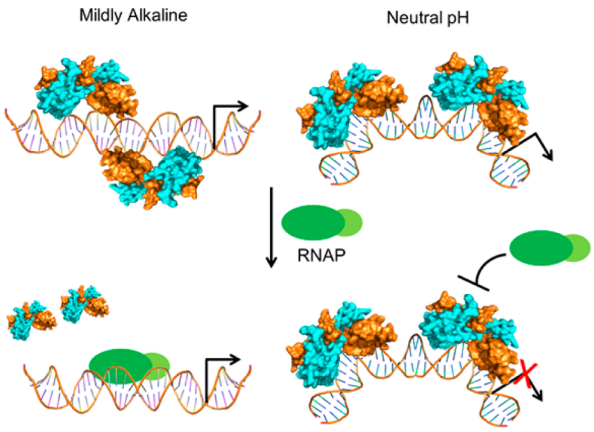



**Figure 8.** PecS-induced DNA conformational changes at neutral pH lead to repression of gene activity. At neutral pH (right), two PecS dimers bind the *pecS*−*pecM* intergenic region and cause a significant DNA distortion that is incompatible with RNA polymerase (RNAP; green) binding to the promoter. At mildly alkaline pH (left), little DNA distortion is induced on PecS binding, allowing RNA polymerase to displace promoter-bound PecS. RNAP not drawn to scale.

That the change in DNA binding and regulatory activity is manifest on increasing pH from neutral to mildly alkaline prompted the consideration that histidine deprotonation might contribute to these observations. H100 in the DNA recognition helix appears to contribute only modestly; its substitution to Phe leads to an apparent decrease in DNA binding affinity and reduced specificity. However, hypersensitive cleavage sites are still present (Figure 6a), suggesting that PecS-H100F induces comparable distortions in the DNA as wild-type PecS. Since H100 is predicted to contact DNA, accessibility of this residue to modification by DEPC is likely to be reduced on DNA binding (Figure 3b).

H142 in α6 is located in a crevice between the dimerization and DNA-binding regions (Figure 1b). Mutation of H142 did not appear to affect affinity or specificity; however, hypersensitive sites were much less pronounced on PecS-H142F binding and similar to the patterns induced by wild-type PecS at pH 8.3 (Figure 5). Consistent with this observation, PecS-H142F was unable to repress the *pecS* promoter *in vivo* (Figure 7d). H142 is predicted to occupy the same position in PecS as it does in the structure of HucR;[23] however, in the PecS model, a glutamate (E146) is predicted to be near H142, in a position to stabilize a positive charge on H142. Such a charge stabilization would be predicted to increase the p$K_a$ of H142, perhaps ensuring that changes in DNA binding are manifest on increasing the pH from neutral to mildly alkaline (and explaining the absence of a similar pH-sensitivity in HucR). We therefore propose that protonation of His142 at neutral pH is associated with a PecS conformation in which DNA distortion is required to accommodate DNA recognition helices. On deprotonation of PecS (or the H142F substitution), such DNA distortion is no longer required for binding.

**Gene Regulation by Urate.** The host plant responds to bacterial infection by production of reactive oxygen species (ROS). Peroxisomes are a major site of intracellular ROS production, and one of the enzymes responsible is xanthine oxidase.[15,16] Xanthine oxidase, which is also found in the cytoplasm, converts hypoxanthine to xanthine and xanthine to urate in the process of generating ROS. While numerous studies have implicated ROS production by xanthine oxidase in stress responses, and the ability of urate to induce arrest of pathogen growth,[33] concentrations of the products xanthine and urate, which would be expected to accumulate transiently and locally in response to infection, have not been estimated under pathological conditions in plants. Urate attenuates the binding of *P. atrosepticum* PecS to the *pecS*−*pecM* intergenic region *in vitro* and *in vivo*, consistent with the inference that it may act as a signaling molecule for the bacterium. Taken together, we propose that derepression of genes under PecS control may be achieved by two mechanisms, a pH-independent association of PecS with urate that results in protein dissociation from cognate DNA and a pH-dependent deprotonation of H142 at mildly alkaline pH that abrogates the PecS-induced conformational changes in promoter DNA that interfere with RNA polymerase recruitment.

## METHODS

**Protein Modeling and Preparation.** The PecS model was generated by homology modeling using the structure of HucR (PDB ID: 2FBK; 31% sequence identity) as a template.[23]

The gene encoding *P. atrosepticum* SCRI 1043 PecS (*ECA_RS10035*) was expressed with an N-terminal $His_6$-tag. Site-directed mutagenesis of PecS was performed by whole plasmid PCR


DOI: 10.1021/acschembio.6b00168
ACS Chem. Biol. 2016, 11, 2049−2056

using the recombinant plasmid containing the PecS gene as a template. Protein was expressed in *E. coli* and purified by metal-affinity chromatography.

**DNA Binding Assays.** For electrophoretic mobility shift assays, the 92-bp (*pecO*) DNA comprising the *pecS*−*pecM* intergenic region was labeled with $^{32}$P. An increasing concentration of PecS was incubated with 0.1 nM $^{32}$P-labeled *pecO* in a binding buffer (50 mM Tris (pH 7.4), 200 mM NaCl, 0.05% Brij58, 20 $\mu$g mL$^{-1}$ BSA, and 4% glycerol). Complex and free DNA were separated by electrophoresis followed by phosphorimaging. Data were fit to the Hill equation $f = f_{max}\ [P]^{n_H}/(K_d + [P]^{n_H})$, where $K_d$ is the apparent equilibrium dissociation constant, $n_H$ is Hill coefficient, $f_{max}$ is the maximal fractional saturation, and [P] is the protein concentration. Data from filter binding assays were processed as described above to determine apparent $K_d$.

DNase I footprinting was performed by fragment analysis.[25] A 317 bp 6-carboxyfluorescein-labeled DNA probe containing the *pecS*−*pecM* intergenic region was used. Binding reactions consisting of 30 ng (14 nM) of fluorescently labeled DNA were incubated with and without protein in binding buffer (50 mM Tris (pH 7.4 or 8.0), 200 mM NaCl, 0.05% Brij58, 20 $\mu$g mL$^{-1}$ BSA, and 4% glycerol), followed by addition of 1 $\mu$L of 10× DNase I reaction buffer and 0.05 U of DNase I.

**Fluorescence Microscopy.** The gene encoding d1EGFP under control of the *pecS* promoter (*pecO*) was cloned into pACYC184. The pACYC184_EGFP_pecO construct and expression vector pET100/D-TOPO (harboring the pecS or the pecS-H142F gene) were cotransformed into *E. coli* Bl21(DE3). The cells were grown at 37 °C to an $OD_{600}$ of 0.5−0.6 with or without IPTG (0.3 mM). Cells were harvested and observed using DIC (differential interference contrast) and fluorescence microscopy. Where required, urate was added to a final concentration of 10 mM. For analysis of the effect of pH on gene expression, cells were grown at pH 8.0 and pH 9.0.

Additional experimental details for all procedures are available in the Supporting Information.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00168.

Figures showing sequence alignment of MarR proteins; conservation of *pecS-pecM* gene pairs; SDS-PAGE gel, gel filtration, and CD spectrum of PecS; DNA binding isotherm; EMSA of complex formation at pH 8.3; filter-binding assay; DNase I footprint of PecS and mutant proteins at different ratios of DNA/protein; EMSA of PecS-H100F; SDS-PAGE gel showing expression of PecS at different pH; expression of EGFP at pH 8.0 and 9.0; attenuation of DNA binding by urate; tryptophan fluorescence spectra of PecS without and with urate; and thermal stability of PecS without and with urate and also supplemental methods (PDF)

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.


### Funding

Supported by the National Science Foundation (MCB-1051610 and MCB-1515349 to A.G.).


### Notes

The authors declare no competing financial interest.


## ■ ACKNOWLEDGMENTS

We thank H. Hale-Donze from the Socolofsky Microscopy Center for assistance with fluorescence microscopy, T. Gauthier for help with CD spectroscopy, and M. Newcomer for the use of FPLC, spectrofluorometer, and spectrophotometer.


## ■ ABBREVIATIONS

DEPC, diethylpyrocarbonate; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; IPTG, isopropyl-$\beta$-D-1-thiogalactopyranoside; MarR, multiple antibiotic resistance regulator; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; UrtR, urate responsive transcriptional regulator

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DOI: 10.1021/acschembio.6b00168
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DOI: 10.1021/acschembio.6b00168
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